ACUTE ASSAY WITH *Daphnia* sp.

1. TEST OBJECTIVE

Definitive Assay - To assess the toxicity of a test material to *Daphnia* and determine the LC50 or EC50 using mortality or immobilization, respectively, as the test end points.

Screening Assay - To assess the toxicity of a test material to *Daphnia* at a single test concentration (e.g., 100 percent effluent or Instream Waste Concentration).

2. TEST ARTICLE

2.1 Description/Identification

Unless otherwise specified, the test material is supplied by the client. Adequate chemical specifications with special reference to hazardous properties and storage conditions is also supplied by the client.

2.2 Methods of Synthesis

In most cases, the test article is an effluent sample. Information on the methods of synthesis, stability, and composition or other characteristics which define the test article are on file with the client.

3. EXPERIMENTAL DESIGN

3.1 Test Organisms

3.1.1 Species

A species of *Daphnia* (water fleas), as determined by project needs, is the test organism.

3.1.2 Source

Daphnia used for acute toxicity tests are obtained from stock cultures maintained in EA's Culture Facility.

3.1.3 Culturing and Holding Conditions

Daphnia cultures are maintained at 20±2°C and a 16-hour light, 8-hour dark photoperiod cycle in an environmentally controlled laboratory. Cultures are maintained in 18.9-L all glass aquaria or other appropriate container and are fed algae (*Selenastrum capricornutum*) and a trout chow/yeast/cereal leaves suspension in the manner described in US EPA (1993). New cultures are initiated on a routine basis to ensure healthy, productive populations. Organisms from cultures producing ephippia are not used for toxicity tests. Certain regulatory or project specific objectives may require organism acclimation to the dilution water when it is different from the holding/culture water.

3.1.4 Age of Test Organisms at Test Initiation

Neonates of known age (i.e., less than 24-hours old) are obtained for testing by segregating adult females from the mass cultures on the day before a test is initiated. On the day of the test, neonates are segregated from the parent organisms.

3.2 Dilution Water

Dilution may be dechlorinated tap water, reconstituted fresh water, or an appropriate receiving water depending on study requirements.

The source of dechlorinated tap water is the City of Baltimore municipal water system. Upon entry to the laboratory, the water passes through a high-capacity, activated-carbon filtration system to remove chlorine and other possible organic contaminants. This water source has proven safe for aquatic organism toxicity testing at EA, as evidenced by maintenance of multigeneration *Daphnia* and *Pimephales promelas* cultures, with no evident loss of fecundity.

3.3 Test Concentration Series

The test concentration series consists of a minimum of five dilutions (e.g., 6.25, 12.5, 25, 50, and 100 percent effluent plus a control) and may be determined from a prior screening of the test material. Rangefinding assays utilize more widely spaced test concentrations and a control. Ambient water or effluent samples may also be evaluated as single concentrations and compared to a control.

3.4 Test Concentration Preparation

Test concentrations are prepared with Class A glassware.

3.5 Test Vessels and Test Volume

Test vessels are 30-ml portion cups or beakers with a 25-ml test volume per test vessel.

3.6 Test Organism Number

Tests are conducted using four replicates per concentration, with five organisms per replicate. Neonates are randomly assigned to each replicate test container. More replicates can be added, if appropriate. A fifth replicate (without organisms) is used for each test concentration to monitor water quality during the test.

3.7 Test Environment

The test vessels are maintained at 20±1°C or 25±1°C (depending on project requirements) in an environmentally controlled laboratory with a 16-hour light, 8-hour dark photoperiod. Temperature within the environmental room is monitored continuously using temperature recorders.

3.8 Analysis of Test Concentrations for Test Article

If required, test solutions may be analyzed for verification of chemical concentrations. The analytical method and number of analyses are determined after consultation with the client. When chemical analyses are necessary, both nominal and actual measured test solution concentrations are reported.

3.9 Test Observations

Each test day, test organisms are observed to record the number of surviving organisms. The study terminates after completion of the observation period (usually 48-hours). The study may be extended, however, at the request of the client.

Each effluent or receiving water sample received is analyzed for temperature, conductivity, alkalinity, hardness, and total residual chlorine. Aliquots of effluent and receiving water may be gently aerated (100 bubbles/min) prior to test initiation if dissolved oxygen is less than 4 mg/L or greater than 100 percent saturation. After test initiation, if the dissolved oxygen in any test chamber is less than 4 mg/L, all test chambers are gently aerated or other corrective action is taken. Water quality measurements recorded on the test solutions daily include dissolved oxygen, pH, temperature and conductivity from a minimum of one replicate of every concentration. To avoid injurying the test organisms, water quality is measured from the fifth replicate test chamber (without organisms). Analytical determinations are conducted according to APHA et al. (1995) and US EPA (1979).

3.10 Solution Renewal (When Applicable)

When static-renewal testing is required, the test solutions are renewed at 24-hours. New test solutions are prepared on the day of renewal. After the new solutions have reached test temperature, the test organisms are transferred from the old test vessels to the new test vessels using a wide bore pipet or glass tube. The number of live organisms is recorded. Caution is given not to stress the test organisms during transfer. After water quality measurements (temperature, pH, dissolved oxygen, and conductivity) are completed, the old solution is discarded.

3.11 Data Analysis

The LC50 or EC50 values and associated statistics are calculated using the probit, moving average, and binomial methods as described by Stephan (1977). Depending on the nature of the data, other methods may be used, including the Trimmed Spearman-Karber method, the probit approximation method of Litchfield and Wilcoxon (1949), SAS probit analysis (SAS Institute 1985) or graphical interpolation using the log concentration vs. percent mortality as described by APHA et al. (1995). The methods used are specified in the final report.

3.12 Test Acceptability

An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests.

4. FINAL REPORT

The final report is prepared to contain at a minimum the following information:

- Objectives and procedures stated in the approved protocol, including any changes made to the original protocol
- Identity of the test article(s) by name or code number and the strength (i.e., quality/purity and a description of any pretreatment)
- Source of the dilution water, its chemical characteristics, and a description of any pretreatment
- Test concentration series used and duration of the assay

- Water quality characteristics of dilution water and selected test concentrations during testing (pH, dissolved oxygen, temperature, etc.)

- Any unforeseen circumstances that may have affected the quality or integrity of the study

- Signature of the project manager, senior technical reviewer, and quality control officer, authorizing release of the report

- Location of all archived data and the original copy of the final report at EA

Items of data to be included in the report consist of experimental design and test performance, effects on general appearance of test organisms (if applicable), morbidity and mortality, presentation of water quality characteristics, and survival data.

5. QUALITY ASSURANCE

5.1 Amendments to Protocol

Amendments to the authorized protocol established by EA or by the client are made only after proper authorization. Such authorization is achieved by completion of the Protocol Amendment Form by EA after consultation with the client.

5.2 Standard Operating Procedures

Unless otherwise specified, all procedures specified in the protocol are subject to detailed Standard Operating Procedures (SOPs) which are contained in the SOP manuals of the participating departments. These SOPs and protocols generally follow the types of requirements as outlined in the U.S. EPA's Good Laboratory Practice Standards (GLPs) (US EPA 1989).

5.3 Reference Toxicant

A reference toxicant test, utilizing sodium dodecyl sulfate (SDS), cadmium chloride, or another appropriate chemical is used as an internal quality check of the sensitivity of the test organisms. Testing is conducted at least once monthly on organisms which are cultured

in-house. The results of each test are compared with historical, species-specific toxicological information from reference toxicant tests performed at EA, to determine if the results are within acceptable limits. Limits are established using the control charts outlined in US EPA (1993).

5.4 Quality Assurance Evaluation

Studies conducted under this protocol may be subject to internal audit by EA's Quality Assurance Unit. A quality control officer is responsible for monitoring each study to assure the client that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with EA's QC program and, if applicable, EPA's GLPs.

5.5 Inspection by Regulatory Authorities

In the event of an inspection of EA by an outside authority during the course of the study, the client whose study is being inspected will be consulted before examiners are permitted access to any of the project records or the experimental areas.

5.6 Archives

Copies of project-specific records shall be transferred to the client promptly after the project is completed or as negotiated and budgeted. Original primary data are retained at EA for 5 years. Primary data include chain-of-custody records, laboratory data sheets, records, memoranda, notes, photographs, microfilm, and computer printouts that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the study report.

5.7 Location

Studies are conducted at the Ecotoxicology Laboratory of EA Engineering, Science, and Technology, Inc. at the Loveton Office in Sparks, Maryland.

6. SPECIFICATIONS OF THE *Daphnia* ACUTE TOXICITY TEST

6.1 Basic References

American Public Health Association (APHA), American Waterworks Association, Water Environment Federation. 1995. Standard Methods for Examination of Water and Wastewater, 19th edition or most recent version. APHA, Washington, D.C.

EA. 1996. Quality Control and Standard Operating Procedures Manual for EA's Ecotoxicology Laboratory. Fifth Revision. EA Manual ATS-102. Internal document prepared by EA's Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Sparks, Maryland.

Litchfield, J.T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharm. Exp. Ther. 96:99-113.

SAS Institute Inc. 1985. SAS User's Guide: Basics, Version 5 Edition. Cary, NC: SAS Institute Inc. 1290 pp.

Stephan, C.E. 1977. Methods for calculating an LC50, in Aquatic Toxicology and Hazard Evaluation (F.L. Mayer and J.L. Hamelink, eds.), pp. 65-84. ASTM STP 634. American Society for Testing and Materials, Philadelphia, Pennsylvania.

US EPA. 1979. Methods for Chemical Analysis of Water and Wastes. EPA/600/4-79/020. U.S. Environmental Protection Agency, Washington, D.C.

US EPA. 1989. Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards. Title 40 CFR Part 792. Fed. Regist. 54(158): 34034-34074.

US EPA. 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

US EPA. 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

6.2 Test Specifications

Test organism:	*Daphnia magna* or *D. pulex*; species to be specified in the study plan and final report
Age:	Less than 24 hours old
Temperature:	20±1°C or 25±1°C
Light quality:	Wide-spectrum fluorescent light
Light intensity:	50-100 f.c.
Photoperiod:	16-hour light, 8-hour dark
Aeration:	None, unless dissolved oxygen falls below 4 mg/L
Dilution water:	Dechlorinated municipal water, reconstituted water, or appropriate receiving water
Test container:	30-ml portion cup or beaker
Test volume:	25 ml per replicate
No. of concentrations:	Definitive assay - Minimum of five test concentrations and a control
	Screening assay - Single test concentration and a control
No. of replicates:	4, with a fifth replicate for monitoring water quality
No. organisms per replicate:	5 (fifth replicate does not contain organisms)
Feeding regime:	Animals will not be fed during test
Test type and duration:	24- to 96-hour acute toxicity test
Endpoints:	Mortality or immobilization
	Immobilization--defined as cessation of movement

except for minor activity of appendages

Mortality--defined as cessation of all movement for a period of at least 5 seconds even when the test container is tapped or rotated, or the organism is gently prodded with glass rod

Test acceptability: 90 percent or greater survival in the control solution.